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PREFACE . . .

VOLUMES IN SE

1. High-Efficie
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2. A Method f
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4. Use of Prim
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: (2) preparative polyacrylamide gel electrophoresis
h methods separate by chain length *and* state of the
ırylated/unphosphorylated) and yield 5′-phosphory-
ɜ of high purity. If, however, the synthetic material,
ırst round of HPLC, is significantly contaminated by
ain length, method (1) is preferable.

lymerase. The "fill-in" protocol (route B), like most
cleotide-directed mutagenesis used to date, requires
reaction *in vitro*. It is known that a purified DNA
h error frequency.[25] Thus, additional mutations out-
ırimer may occasionally be expected. Such events
ıserved and, for this reason, it is important to se-
A segment under study after mutagenesis. Conceiv-
transfect" protocol (route A), which leaves all enzy-
ıe transfected cell, may be more accurate in that
ıowever, the body of experience with route A is not
eaningful comparison.
ıe of the Transfection Host. Escherichia coli strains
ɜmatch repair (such as the mutS strain used in this
eased frequency of spontaneous mutations (mutator
ɜible, in principle, that additional and unwanted mu-
uced into the target DNA during propagation of the
age. This, however, is of no practical significance,
ıtaneous mutation is still lower by several orders of
equency of the constructed mutation.
ɜnation of Cytosine Residues. The construction of
ing of the DNA solution to 100°. Under these condi-
ıination of 2′-deoxycytidine residues to 2′-deoxyuri-
ɔant reaction. Such reactions would be detrimental
provide start points of unwanted repair synthesis
ɜ double-stranded portion of the gdDNA. More seri-

ıd W. Werr, *in* "Chemical and Enzymatic Synthesis of Gene
ɜen and A. Lang, eds.), p. 199. Verlag Chemie, Weinheim, Fed-
ɣ, 1982.
ɜh, *in* "Oligonucleotide Synthesis: A Practical Approach" (M. J.
ess, Oxford, England, 1984.
ınna, F. Georges, and S. Narang, *in* "Oligonucleotide Synthesis:
M. J. Gait, ed.), p. 135. IRL Press, Oxford, England, 1984.
Knill-Jones, *J. Mol. Biol.* **165**, 633 (1983).

ously, a C/G to T/A transition would be induced at the site of such a lesion when it was located within the single-stranded gap. In one out of many dozens of cases studied (route B), we have found an additional C/G to T/A transition. It is, of course, not possible to decide whether this mutation was due to the described cytosine deamination or to an error of the DNA polymerase (see above). Again, route A may offer an advantage due to the expected *in vivo* killing of entering gdDNA molecules that carry a 2′-deoxyuridine residue in their single-stranded part.

Flexibility

The applicability of the gdDNA method reaches beyond oligonucleotide-directed mutation construction. Gapped duplex DNA molecules are also ideal substrates for other methods of directed mutagenesis such as forced gap misrepair[26] or attack by single-strand selective chemicals (e.g., bisulfite[27]). Studies in "reversed genetics" can be planned in such a way that a stock of gdDNA is prepared and the last two (or similar) methods are used for saturation of the gap sequence with (single) point mutations in order to identify functionally critical points within the cloned gene or regulatory DNA segment. Once these are known, the same original gdDNA can be used to ask specific questions via oligonucleotide-directed construction of predetermined mutations.

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[19] Rapid and Efficient Site-Specific Mutagenesis without Phenotypic Selection

By THOMAS A. KUNKEL, JOHN D. ROBERTS, and RICHARD A. ZAKOUR

The deliberate alteration of DNA sequences by *in vitro* mutagenesis has become a widely used and invaluable means of probing the structure and function of DNA and the macromolecules for which it codes. In an ideal experiment, alterations are produced at high efficiency and with minimum effort; such features become especially important when sequence changes produce silent, unknown, or nonselectable phenotypes. To overcome some of the limitations that lead to low efficiency, several

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variations of *in vitro* mutagenesis techniques have been developed.[1-5] Each procedure has its own advantages, but each also requires additional time and/or technical expertise. An alternative method[6] is presented here that is simple, rapid, and efficient. This method takes advantage of a strong biological selection against the original DNA template which is preferentially destroyed on transfection. The use of this special template can be combined with many of the previously described *in vitro* mutagenesis methods. What we describe here is not, therefore, a new procedure for site-directed mutagenesis but is, rather, the use of standard and well-established procedures in conjunction with an unusual template. This combination permits flexibility in the choice of mutagenesis techniques and makes possible the highly efficient recovery of mutants.

Principle

The basis of this method is the performance of site-directed mutagenesis using a DNA template which contains a small number of uracil residues in place of thymine.[6] The uracil-containing DNA is produced within an *Escherichia coli dut⁻ ung⁻* strain. *Escherichia coli dut⁻* mutants lack the enzyme dUTPase[7,8] and therefore contain elevated concentrations of dUTP which effectively competes with TTP for incorporation into DNA. *Escherichia coli ung⁻* mutants lack the enzyme uracil *N*-glycosylase[9] which normally removes uracil from DNA.[10] In the combined *dut⁻ ung⁻* mutant, uracil is incorporated into DNA in place of thymine and is not removed.[11-13] Thus, standard vectors containing the sequence to be changed can be grown in a *dut⁻ ung⁻* host to prepare uracil-containing DNA templates for site-directed mutagenesis.

For the *in vitro* reactions typical of site-directed mutagenesis protocols, uracil-containing DNA templates are indistinguishable from normal templates. Since dUMP in the template has the same coding potential as TMP,[14] the uracil is not mutagenic, either *in vivo* or *in vitro*. Furthermore, the presence of uracil in the template is not inhibitory to *in vitro* DNA synthesis. Thus, this DNA can be used *in vitro* as a template for the production of a complementary strand that contains the desired DNA sequence alteration but contains only TMP and no dUMP residues.

After completing the *in vitro* reactions, uracil can be removed from the template strand by the action of uracil *N*-glycosylase.[10] Glycosylase treatment can be carried out with purified enzyme[6] but is most easily achieved by transfecting the unfractionated products of the *in vitro* incorporation reaction into competent wild-type (i.e., *ung⁺*) *E. coli* cells. Treatment with the glycosylase, either *in vitro* or *in vivo*, releases uracil-producing apyrimidinic (AP) sites only in the template strand.[15] These AP sites are lethal lesions, presumably because they block DNA synthesis,[13,16,17] and are sites for incision by AP endonucleases which produce strand breaks.[15] Thus, the template strand is rendered biologically inactive, and the majority of progeny arise from the infective[6,18-20] complementary strand which contains the desired alteration. The resulting high efficiency of mutant production (typically >50%) allows one to screen for mutants by DNA sequence analysis, thus identifying mutants and confirming the desired alteration in a single step. This feature is particularly advantageous when no selection for the desired mutants is available.

Materials and Reagents

Bacterial Strains

The *E. coli* strains and their sources are listed in Table I. Their use and maintenance are described below.

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TABLE I
Escherichia coli STRAINS

Strain designation	Genotype	Source
BW313	HfrKL16 PO/45 [*lysA*(61-62)], *dut1*, *ung1*, *thi1*, *relA1*	*a*
CJ236[b]	*dut1*, *ung1*, *thi1*, *relA1*/pCJ105 (Cm^r)	*c*
RZ1032	As BW313, but Zbd-279::Tn*10*, *supE44*	*d*
NR8051	[Δ(*pro–lac*)], *thi*, *ara*	*a*
NR8052	[Δ(*pro–lac*)], *thi*, *ara*, *trpE9777*, *ung1*	*a*
KT8051	[Δ(*pro–lac*)], *thi*, *ara*/F′ (*proAB*, $lacI_q^-Z^-$ ΔM15)	*e*
KT8052	[Δ(*pro–lac*)], *thi*, *ara*, *trpE9777*, *ung1*/F′ (*proAB*, $lacI_q^-Z^-$ ΔM15)	*e*
CSH50	[Δ(*pro–lac*)], *thi*, *ara*, *strA*/F′ (*proAB*, $lacI_qZ^-$ ΔM15, *traD36*)	*f*
NR9099	[Δ(*pro–lac*)], *thi*, *ara*, *recA56*/F′ (*proAB*, $lacI_q^-Z^-$ ΔM15)	*g*

[a] As described by T. A. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 488 (1985), but see "Uses, Maintenance, and Characteristics of Bacterial Strains" in this chapter.
[b] The plasmid pCJ105 (Cm^r) was constructed as described by C. M. Joyce and N. D. F. Grindley, *J. Bacteriol.* **158,** 636 (1984).
[c] C. M. Joyce, Yale University, New Haven, Connecticut.
[d] See text.
[e] K. Tindall, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.
[f] T. A. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 1494 (1984).
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Growth Media

YT medium: Bactotryptone, 8 g; Bactoyeast extract, 5 g; NaCl, 5 g. Add to 1 liter of H_2O and sterilize in an autoclave.

2× YT medium: Bactotryptone, 16 g; Bactoyeast extract, 10 g; NaCl, 10 g; pH adjusted to 7.4 with HCl. Add H_2O to 1 liter and sterilize in an autoclave.

Soft agar: NaCl, 9 g; Difco agar, 8 g. Add H_2O to 1 liter and sterilize in an autoclave.

VB salts (50×): $MgSO_4 \cdot 7H_2O$, 10 g; citric acid (anhydrate), 100 g; K_2HPO_4, 500 g; $Na_2HPO_4 \cdot 2H_2O$, 75 g. Dissolve the above in 670 ml dH_2O, bring volume to 1 liter, and sterilize in an autoclave. After dilution, pH is 7.0–7.2.

Minimal plates: Add 16 g of Difco agar to 1 liter of dH_2O and sterilize in an autoclave. When the agar has cooled to 50°, add 0.3 ml of 100 m*M* IPTG, 20 ml of 50× VB salts, 20 ml of 20% glucose, and 5 ml of 1 mg/ml thiamine–HCl. [Each of these solutions was sterilized either by filtration (0.2-μm pore) or in an autoclave prior to their addition to the 50° agar.] The mixture is mixed well and dispensed into sterile petri dishes (30 ml/plate).

Enzymes and Reagents

T4 DNA polymerase and T4 polynucleotide kinase were from Pharmacia, Molecular Biology Division. T4 DNA ligase was from New England Biolabs or International Biotechnologies, Inc. Deoxynucleoside triphosphates (HPLC grade, 100 m*M* solutions) were purchased from Pharmacia, Molecular Biology Division, and used without further purification. 5-Bromo-4-chloroindolyl-β-D-galactoside (Xgal) was from Bachem Chemicals. Isopropylthio-β-D-galactoside (IPTG) was from Bethesda Research Laboratories. Phenol (ultrapure grade) was obtained from Bethesda Research Laboratories or International Biotechnologies, Inc., and used without further purification. All other chemicals were obtained from standard suppliers of molecular biological reagents.

Stock Solutions

Xgal: 50 mg/ml in *N,N*-dimethylformamide (DMF), stored at −20°. Avoid exposure to light.

IPTG: 24 mg/ml in dH_2O, stored at −20°.

PEG/NaCl (5×): Polyethylene glycol 8000, 150 g; NaCl, 146 g. Dissolve in dH_2O, adjust volume to 1 liter, and filter sterilize using a 0.2-μm filter.

Phenol extraction buffer (PEB): 100 m*M* Tris–HCl (pH 8.0); 300 m*M* NaCl; 1 m*M* EDTA.

Phenol: equilibrated versus multiple volumes of PEB until the pH of the aqueous phase is ~8.0, stored in a brown bottle at 4°.

TE buffer: 10 m*M* Tris–HCl (pH 8.0); 0.1 m*M* EDTA.

Kinase buffer (10×): 500 m*M* Tris–HCl (pH 7.5); 100 m*M* $MgCl_2$; 50 m*M* dithiothreitol.

SSC (20×): 3 *M* NaCl; 300 m*M* sodium citrate.

SDS dye mix (10×): 10% sodium dodecyl sulfate; 1% bromophenol blue; 50% glycerol.

TAE buffer (50×): Tris base, 242 g; glacial acetic acid, 57.1 ml; EDTA, 100 ml of a 500 m*M* solution (pH 8.0). Dissolve in dH_2O, adjust volume to 1 liter.

Methods

Uses, Maintenance, and Characteristics of Bacterial Strains

Uracil-containing DNA was first prepared as a template for *in vitro* mutagenesis as described by Sagher and Strauss[13] using *E. coli* strain BW313. This strain was chosen on the basis of three criteria which are crucial for the successful production of uracil-containing viral DNA tem-

plates: (1) susceptibility to infection by small filamentous bacteriophages (e.g., M13), which requires the F (sex factor) pilus; (2) the presence of the Dut⁻ and Ung⁻ phenotypes, which are required for the stable incorporation of uracil into phage DNA; and (3) a low rate of spontaneous mutation in the progeny phage, so that unwanted mutations are not introduced into the DNA target.

In the original publication of this method,[6] BW313 was incorrectly described as F′*lysA*. BW313 is actually an Hfr strain with the integrated F factor providing the pilus needed for phage attachment. Since there is no selective pressure that can be used to maintain the Hfr phenotype, we store BW313 frozen (−70°) in multiple aliquots containing 3 ml of a mid-log culture (2×10^8 to 2×10^9 cells/ml) mixed with 0.3 ml of DMSO. When needed, a vial is thawed and cells are streaked on a YT plate (or any rich medium plate) to obtain single colonies. This plate may be used as a source of colonies for over 2 months when stored at 4°. We have not encountered problems in achieving M13 infections using this procedure. However, since others have observed a loss of infectability with this strain, a second strain was produced by introducing a selectable F′ that confers resistance to chloramphenicol into a BW313 strain that had lost its competence for M13 infection. This strain (CJ236, produced by Catherine Joyce at Yale) stably retains susceptibility to M13 infection when selective pressure is maintained, and it has been used successfully by us to prepare uracil-containing DNA for several site-directed mutagenesis experiments.

Since BW313 does not contain an amber suppressor, a third *E. coli dut⁻ ung⁻* strain was constructed for use with cloning vectors which contain amber mutations in essential genes (e.g., M13 mp8). Strain JM101 (*supE44*) was transduced to tetracycline resistance using P1 grown on strain SK2255 which carries the *tet*ʳ marker on a transposable element, Zbd-279::Tn*10*. A tetracycline-resistant derivative of JM101 was used to grow P1 which were then used to transduce strain BW313 to tetracycline resistance. The BW313 Tetʳ transductants were tested for their ability to support growth of phage M13mp8 (which contains two amber mutations) and for the ability to produce M13mp2 phage containing uracil in their DNA (due to the host *dut1* and *ung1* mutations). The resulting Hfr strain (RZ1032) fulfills these criteria and templates prepared from this strain perform well in subsequent *in vitro* mutagenesis experiments. The *supE44* is maintained with tetracycline selection. This strain grows somewhat more slowly than BW313, and characteristically produces smaller M13 plaques and lower phage yields in liquid cultures.

As with BW313, RZ1032 loses its Hfr phenotype at a low but bothersome frequency and a single-colony isolate may not support M13 infection. (At present, a selectable F′ has not been placed into RZ1032.) To overcome this problem with either BW313 or RZ1032, several individual colonies should be picked from a plate and liquid cultures (YT medium), each from a single colony, should be screened by plaque assays on plates to test for M13 infectability. Once a competent culture is identified, aliquots can be stored indefinitely at −70° in 10% DMSO. We have not encountered instability of the *dut⁻* and *ung⁻* markers in BW313, CJ236, or RZ1032 when they are grown in rich medium. Likewise, beyond the usual slight increase in mutation frequency associated with *dut⁻ ung⁻* strains (see below), which is negligible in site-directed mutagenesis protocols, we have observed neither high mutation frequencies nor spurious mutations on growing vectors in these strains.

The other strains listed in Table I have been useful in establishing the utility of uracil-containing DNA, but are not required for most mutagenesis protocols. NR8052 (*ung⁻*) and its wild-type (*ung⁺*) parent, NR8051, can be used to measure the relative survival of uracil-containing DNA upon transfection. Similarly, the newly constructed derivatives of these strains, prepared by Kenneth Tindall of the National Institute of Environmental Health Sciences and designated KT8052 and KT8051, respectively, can be used for a similar analysis of intact phage since they contain an F′. Survival data can be obtained by comparing phage titers on BW313 (or CJ236 or RZ1032, all *ung⁻* strains) with titers on any wild-type (*ung⁺*) strain. However, NR8052 and NR8051 have another advantage in that they can also be used to determine spontaneous mutation rates in the phage-borne *lacZα* gene by following the loss of α-complementation as previously described.[6,17] Such experiments were carried out to establish that growth in the *dut⁻ ung⁻* host is not mutagenic.[6]

Escherichia coli CSH50 and NR9099 are *ung⁺* and are routinely used for α-complementation experiments in our laboratory. These, as well as other *ung⁺ E. coli* strains (for example, the JM series of α-complementation strains), are acceptable hosts for transfection of the products of the *in vitro* mutagenesis reactions with uracil-containing templates.

Growth of Phage

Uracil-containing viral DNA template is isolated from intact M13 phage grown on an *E. coli dut⁻ ung⁻* strain. Phage can be produced as previously described[6] or by a simpler method which we present here. Using a sterile pipet tip, remove one plaque [usually 10^9–10^{10} plaque-forming units (pfu)/plaque for M13] from a plate and place it in 1 ml of sterile YT medium in a 1.5-ml Eppendorf tube. Incubate the tube for 5 min at 60° to kill cells, vortex vigorously to release the phage from the agar,

then pellet cells and agar with a 2-min spin in a microcentrifuge. Place 100 μl of the resulting supernatant (containing 10^8–10^9 pfu) into a 1-liter flask containing 100 ml of YT medium supplemented with 0.25 μg/ml uridine; we have found that neither the thymidine nor the adenosine supplementation originally described[6] is necessary, since omitting these effected neither phage yield nor mutation frequency. Add 5 ml of a mid-log culture of the appropriate *E. coli dut⁻ ung⁻* strain. These proportions result in a multiplicity of infection of ≤1. Most or all phage infect cells and are thus "passaged" through the *dut⁻ ung⁻* strain. Since few uracil-lacking phage remain, a single cycle of growth results in a sufficient survival difference (as measured by titers on *ung⁺* and *ung⁻* hosts) to make the DNA suitable for the *in vitro* mutagenesis protocol.

The flask is incubated with vigorous shaking at 37°. We have prepared phage from cultures incubated for as short as 6 hr or as long as 24 hr. Shorter times are recommended for vectors that contain unstable inserts, since this will help to avoid the growth advantage of phages which have deleted the insert. (A *recA⁻* derivative of a *dut⁻ ung⁻* strain might be useful to stabilize otherwise unstable DNA sequences, but at present we do not have such a strain.)

After incubation at 37°, the culture is centrifuged at 5000 *g* for 30 min. The clear supernatant contains the phage at about 10^{11} pfu/ml. (This yield may vary depending on the vector and strain used; our experience with RZ1032 suggests that phage titers of 2–5 × 10^{10} pfu/ml are not unusual.) Before preparing viral template DNA, the phage titers should be compared on *ung⁻* and *ung⁺* hosts. Phage which contain uracil in the DNA have normal biological activity in the *ung⁻* host but greater than 100,000-fold lower survival in the *ung⁺* host. Phage produced in the *dut⁻ ung⁻* host show only a slight (~2-fold) increase in mutation frequency when compared to phage produced in wild-type *E. coli*. Loss of α-complementation occurs in about 0.1% of the uracil-containing phage,[6] a negligible background compared to frequencies of 50–90% in site-directed mutagenesis experiments.

Preparation of Template DNA

Phage are precipitated from the clear supernatant by adding 1 volume of 5× PEG/NaCl to 4 volumes of supernatant, mixing, and incubating the phage at 0° for 1 hr. The precipitate is collected by centrifugation at 5000 *g* for 15 min, and the well-drained pellet is resuspended in 5 ml of PEB in a 15-ml Corex tube. After vigorous vortexing, the resuspended phage solution is placed on ice for 60 min and then centrifuged as above to remove residual debris. (This step has proved useful in reducing the level of

endogenous priming in subsequent *in vitro* DNA polymerase reactions.) The supernatant containing the intact phage is extracted twice with phenol (previously equilibrated with PEB) and twice with chloroform : isoamyl alcohol (24 : 1). The DNA is precipitated [by adding 0.1 volume of 3 *M* sodium acetate (pH 5.0) and 2 volumes of ethanol and chilling the mixture to −20°], collected by centrifugation and resuspended in TE buffer. The DNA concentration is determined spectrophotometrically at 260 nm using an extinction coefficient of 27.8 ml/mg cm (i.e., 1 OD_{260} = 36 μg/ml) for single-stranded DNA. The purity of the DNA is examined by agarose gel electrophoresis as described below, overloading at least one lane to visualize trace contaminants.

We have found no need to further purify the DNA in order to achieve high efficiencies of *in vitro* mutagenesis. If problems related to template purity are encountered, or if mutant production approaching 100% is needed, the DNA can be subjected to any standard purification procedure, since the substitution of a small percentage of thymine residues by uracil should not affect the physical properties of the DNA.

In principle, any cloning vector that can be passaged through an *E. coli dut⁻ ung⁻* strain can be used with the uracil selection technique. Once the uracil-containing DNA is prepared, it can be used as would be any standard template, in a variety of *in vitro* methodologies for altering DNA sequences.[1] We present below a typical oligonucleotide-directed mutagenesis experiment to make several points of interest. Some of these have previously appeared in the literature (see Smith[1] and references therein, as well as several other chapters in this volume[2–4]) and are well known to investigators acquainted with this field. However, these notes may be useful to those less familiar with *in vitro* mutagenesis.

Example of the Method

For reasons to be published elsewhere, we required a mutant of M13mp2 containing an extra T residue in the viral-strand run of four consecutive Ts at positions 70 through 73 (where position 1 represents the first transcribed base) in the coding sequence for the α peptide of β-galactosidase.[21]

Description and Phosphorylation of the Oligonucleotide

A 22-base oligonucleotide, complementary to positions 62–82 and containing an extra (i.e., fifth) A residue, was purchased from the DNA

[21] T. A. Kunkel, *J. Biol. Chem.* **260**, 5787 (1985).

Synthesis Service, Dept. of Chemistry, Univ. of Pennsylvania. (The ability of this or any oligonucleotide to prime *in vitro* DNA synthesis at the appropriate position should be examined.[1]) The 5′ OH of the 22-mer was phosphorylated (for subsequent ligation) in a 20-μl reaction containing 50 m*M* Tris–HCl (pH 7.5), 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 1 m*M* ATP, 2 units of T4 polynucleotide kinase, and 9.0 ng of the oligonucleotide. The reaction was incubated at 37° for 1 hr and terminated by adding 3 μl of 100 m*M* EDTA and heating at 65° for 10 min.

Hybridization of the Oligonucleotide to the Uracil-Containing Template

To the phosphorylated oligonucleotide was added 1 μg (in 0.6 μl) of single-stranded, uracil-containing, circular wild-type M13mp2 DNA and 1.2 μl of 20× SSC. After mixing and spinning the sample briefly (5 sec) in a microcentrifuge, the tube was placed in a 500-ml beaker of water at 70° and allowed to cool to room temperature. After another 5-sec centrifugation to spin down condensation, the tube was placed on ice.

We typically perform hybridization at a primer : template ratio between 2 : 1 and 10 : 1 since higher ratios do not yield more of the desired product and in some cases inhibit ligation. Hybridization conditions should be chosen to optimize heteroduplex formation with the particular oligonucleotide and template being used, and these conditions are expected to vary widely depending on the resulting heteroduplex (see Smith[1] and references therein for more details).

In Vitro DNA Synthesis and Product Analysis

The sequence contained within the oligonucleotide is converted to a biologically active, covalently closed circular (CCC) DNA molecule by DNA synthesis and ligation. These reactions are performed in a volume of 100 μl containing 20 μl of the above hybridization mixture; 20 m*M* HEPES (pH 7.8); 2 m*M* dithiothreitol; 10 m*M* $MgCl_2$; 500 μ*M* each of dATP, TTP, dGTP, and dCTP; 1 m*M* ATP; 2.5 units (as defined by the supplier) of T4 DNA polymerase; and 2 units of T4 DNA ligase. All components are mixed at 0° (enzymes being added last), and the reaction is incubated at 0° for 5 min. The tube is placed at room temperature for 5 min, then at 37° for 2 hr. The rationale for this pattern of incubation is as follows.

The reaction is begun at lower temperatures (0°, then room temperature) to polymerize a small number of bases onto the 3′ end of the oligonucleotide, thus stabilizing the initial duplex between the template phage DNA and the mutagenic oligonucleotide primer. However, since T4 DNA polymerase does not utilize long stretches of single-stranded DNA template well at low temperature, synthesis is then completed at 37°. We have not encountered significant pausing by T4 DNA polymerase under these conditions. The high concentration of dNTPs (500 *mM*) serves to optimize DNA synthesis and to reduce the 3′-exonuclease activity of the T4 DNA polymerase.

The reaction is terminated by addition of EDTA to 15 m*M* (3 μl of a 500 m*M* stock). The products of the reaction are then examined by subjecting 20 μl (to which is added 2.5 μl of SDS dye mix) to electrophoresis in a 0.8% agarose gel (in 1× TAE buffer containing 0.5 μg/ml ethidium bromide). For comparison, an adjacent lane should contain the appropriate standards: single-stranded, circular viral DNA, and double-stranded replicative form I (supercoiled CCC) and form II (nicked circular) DNAs.

The product of the *in vitro* DNA synthesis reaction should migrate at the same rate as the RF I standard, indicating that the DNA has been converted from primed circles to RF IV (duplex, CCC relaxed DNA) by the combined action of DNA polymerase and ligase. (Note that, in gels containing ethidium bromide, the dye will bind to the CCC relaxed DNA, generating positive supercoils and causing the RF IV to migrate like RF I.) Our experiments typically yield 80% conversion to primarily double-stranded DNA, but only 10–50% of this material is ligated to form covalently closed circles. Lower quality enzymes, unusual inserts, contaminants, or less than optimum reaction conditions may reduce the yield. (For more comments on the DNA synthesis step, see Troubleshooting below.)

Transfection and Plating

After incorporation of the oligonucleotide into double-stranded DNA *in vitro*, the DNA can be used to transfect (or transform) any competent *E. coli* strain (prepared with either $CaCl_2$[22] or by the method of Hanahan[23]). Provided the strain is *ung*$^+$, one can take advantage of the selection against the uracil-containing template strand. Unless high biological activity is required, the products of the *in vitro* DNA synthesis reaction can be used directly without further manipulation to remove reaction components.

For the reaction described above, 10 μl of the DNA synthesis mixture (containing approximately 80 ng of the double-stranded DNA) was added to 1 ml of competent CSH50 cells (prepared according to Hanahan[23]) in a sterile glass tube. A second transfection with a known amount of RF

[22] A. Taketo, *J. Biochem.* **72,** 973 (1972).
[23] D. Hanahan, *J. Mol. Biol.* **166,** 557 (1983).

DNA was performed to determine the transfection efficiency. The cells were gently mixed and incubated on ice for 30 min, heat shocked at 42° for 2 min, then returned to ice. Small volumes of this mixture (1, 5, 10, 50, or 100 μl) were added to tubes containing 2.5 ml of soft agar (at 50°), 2.5 mg Xgal (previously mixed well with the media to disperse the DMF), 0.24 mg IPTG, and 0.2 ml of mid-log culture of CSH50 cells. The mixtures were poured onto minimal plates and allowed to solidify. Plates were incubated at 37° overnight to allow blue color to develop as a measure of α-complementation. Nonconfluent plates (in this case the 1- and 5-μl plates) were scored for total plaques. Transfection using 10% (~80 ng in 10 μl) of the *in vitro* DNA synthesis reaction produced 193,000 plaques, an efficiency of about 2400 pfu/ng. In this particular instance, the desired mutants are expected to be colorless due to the addition of an extra base in the *lacZα* coding region. Colorless plaques comprised 70% of the total.

To confirm that the colorless mutants contained the expected sequence alteration, DNA from several phage was prepared for sequencing. First, 10 colorless plaques were harvested into 0.9% NaCl, serially diluted, and replated (as above) to obtain plaques derived from single phage particles. This genetic purification eliminates the possibility that the DNA to be sequenced comes from a plaque which contained two genotypes (as is possible in the original plaques) due either to transfection by a heteroduplex DNA molecule or to plaque overlap. This step becomes unnecessary if, following heat shock of the transfection mixture, one adds rich medium and continues incubation at 37° to allow production of progeny phage, which can then be diluted and plated. We have not used this extra incubation in experiments described here; thus, quantitation of the efficiency of mutant production does not require a correction for differences in growth rates of mutant versus wild-type phages. In either instance, once a purely mutant shock is obtained, the DNA can be sequenced by standard techniques. In our example, all 10 colorless plaques contained the expected change, an extra T residue in the viral strand.

Troubleshooting

Unsuccessful experiments are usually characterized by one of two outcomes. The first is low biological activity on transfection. To eliminate the obvious possibility that the *E. coli* cells were not competent, we always perform a parallel transfection with a known quantity of normal DNA to determine the transfection efficiency. Since biological activity depends on a complete complementary strand, inefficient DNA synthesis may also lead to low numbers of plaques. When this problem appears, a careful product analysis is warranted. In our experience, the presence of completely double-stranded DNA in the transfection mix has always been

a harbinger of good biological activity. However, even when the yield of fully double-stranded DNA is poor, the strong selection against incompletely copied or uncopied uracil-containing templates which occurs on transfection permits the desired mutant to be recovered. In fact, we have obtained the desired sequence alterations from transfection of reactions that contained no double-stranded DNA as judged by agarose gel electrophoresis. These reactions yielded very few plaques, but 40% of the surviving DNA carried the mutant sequence.

In two instances (out of ~50 separate experiments), however, we observed no biological activity from mixtures which exhibited a band of DNA at the position of RF I on agarose gel electrophoresis in the presence of ethidium bromide. In these two cases, a further analysis was performed to determine the nature of the reaction products. If the product band were indeed the desired species, that is covalently closed circles of RF IV, this DNA would migrate like RF II DNA in the absence of the intercalating dye. Another possibility is that the product was the result of incomplete synthesis of the complementary strand and that its migration at the position of RF I in the initial agarose gel was fortuitous. If this were true, the migration of this DNA would not be affected by ethidium bromide. In both cases of low biological activity, the synthesis products migrated at the position of RF I in both the presence and absence of ethidium bromide, indicating that DNA synthesis was incomplete and explaining the lack of biological activity. (In both cases, the incomplete synthesis was due to the use of an old T4 DNA polymerase preparation that was no longer fully active.)

Incomplete synthesis can result from several factors, including inefficient hybridization of the oligonucleotide primer, inactive (or excess) DNA polymerase, contaminants in the DNA, the polymerase, or the reagents, or a DNA template which contains structures (e.g., hairpin loops) that block polymerization. (For example, with several T4 DNA polymerase preparations we have found as much as 10 units of enzyme may be required to achieve complete synthesis.) Such problems must be dealt with on an individual basis, e.g., by varying hybridization conditions, by ensuring the activity of the DNA polymerase, by repurifying the DNA, or by using alternative incubation temperatures or single-stranded DNA binding protein to assist the polymerase in synthesis on unusually difficult templates.

Low biological activity could also result from dNTP contamination by dUTP (e.g., by deamination of dCTP), which, when incorporated *in vitro*, provides targets for the production of lethal AP sites.[24] For this reason,

[24] P. D. Baas, H. A. A. M. van Teeffelen, W. R. Teerstra, H. S. Jansz, G. H. Veeneman, G. A. van der Marel, and J. H. van Boom, *FEBS Lett.* **110**, 15 (1980).

high quality dNTP substrates should be used to eliminate the need for dUTPase treatment of the deoxynucleoside triphosphates.[6,14] (dUTPase is not commercially available.)

The second undesirable outcome is a low percentage of mutants among the progeny. To confirm that there is indeed strong selection for the mutant strand over the template strand due to uracil residues in the latter, a titer of the phage from which the template is purified should be performed on *ung*$^-$ and *ung*$^+$ hosts. The difference in titers should be greater than 100,000-fold, although phage that show a smaller difference will still yield mutants at high efficiencies.[6] If a large difference in titer is not obtained, there is insufficient uracil in the viral DNA and new templates should be prepared. (Another but less likely possibility is that the putative *ung*$^+$ host is genetically and/or phenotypically *ung*$^-$.)

A low percentage of mutants can also result from impurities in the template DNA which provide endogenous primers for complementary strand synthesis. The amount of endogenous priming can be determined by performing a DNA synthesis reaction without added oligonucleotide and examining the products by gel electrophoresis. When template is prepared as described above, the amount of RF IV DNA produced *in vitro* should be negligible in the absence of oligonucleotide. With impure template preparations, essentially all the single-stranded circular DNA can be converted to double-stranded product without added primer, in which case biological activity but no mutants will be obtained. The impurities can be removed by standard techniques (e.g., alkaline gradients) or the template can be prepared anew.

Another source of low mutant yield is displacement of the oligonucleotide during *in vitro* DNA synthesis of the strand which carries the mutation (see line 4, Table 2, in Ref. 6). This possibility is not a concern when one uses T4 DNA polymerase, since this enzyme does not perform strand displacement synthesis under the conditions given above. However, such problems may arise with some primers and/or templates that have more, or stronger, polymerase pause sites than do our templates. In these situations, the polymerase can be assisted by its homologous single-stranded DNA binding protein (the T4 gene *32* protein). Alternatively, the Klenow fragment of *E. coli* DNA polymerase I, which efficiently synthesizes complete complementary strands without significant pausing, can be used at low temperature (to prevent displacement of the oligonucleotide). Even this enzyme has difficulty with some templates at reduced temperatures; in these instances *E. coli* single-stranded DNA binding protein may be helpful.

One pathway by which the mutant, complementary strand of DNA may be selected against is the methyl-directed mismatch correction system present in most *E. coli* strains.[25] Repair synthesis which uses the uracil-containing strand as a template before it is destroyed will eliminate the artificially produced mutation from the complementary strand. Although this process is not a major concern (since we routinely achieve high efficiency, 50–70%), two means of reducing mismatch repair and improving mutant production can be employed in those situations where one desires the highest efficiency possible. The first is to transfect the DNA into competent cells made from *E. coli* mutator strains (*mutH*, *mutL*, or *mutS*) which are deficient in mismatch correction.[25] [In a typical experiment, the efficiency of mutant production was improved from 51% (wt) to 57% (*mutH*), 67% (*mutL*), and 59% (*mutS*).] A second strategy is to treat the product DNA with uracil *N*-glycosylase as described[6] to form AP sites in the template strand, followed by alkali treatment to hydrolyze the phosphodiester bonds at AP sites and to disrupt hydrogen bonding. This protocol eliminates the transforming activity of the parental strand and leaves only the covalently closed, single-stranded DNA which contains the desired sequence alteration as a source of transforming DNA. In practice, this treatment improved efficiency from 51 to 89%.[6] We do not routinely utilize either mutator strains or glycosylase, since, for our purposes, 50–70% efficiency is more than sufficient and since uracil *N*-glycosylase is not commercially available.

We originally observed good biological activity but low efficiency of mutagenesis when ligase was omitted from the *in vitro* reaction (see line 4, Table 2, in Ref. 6). This observation was made with DNA products synthesized by the DNA pol I Klenow fragment under conditions that allow strand displacement (37°). More recently, using T4 DNA polymerase as described above, we attempted oligonucleotide-directed mutagenesis of the gene for the *E. coli* cyclic AMP receptor protein. We used a 36-mer which primed DNA synthesis efficiently but could not be ligated to form CCC DNA (i.e., only RF II but no RF IV DNA was produced), perhaps because of abberant chemistry during oligonucleotide synthesis or impurities in the oligonucleotide preparation. Despite the fact that the product DNA was not covalently closed, the mutant yield, with no selection, was 67% (six of the nine clones sequenced having the appropriate mutation). We conclude that, with T4 DNA polymerase (i.e., in the absence of strand displacement), ligation *in vitro* is not required for highly efficient mutant production. Presumably, on transfection, the cell performs the necessary processing at the termini before the template DNA is destroyed.

Lest these comments on troubleshooting discourage use of the technique, we reiterate that, after the preparation of the uracil-containing

[25] B. W. Glickman and M. Radman, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1063 (1980).

template, oligonucleotide-directed mutagenesis at or above 50% efficiency is a simple procedure, consisting of a polymerization reaction and a transfection.

Variations

We have presented here a simple oligonucleotide-directed mutagenesis protocol to demonstrate the utility of the uracil selection technique for generating mutants with high efficiency (≥50%). Uracil-containing DNA can be prepared for any vector that can be passaged through an *E. coli* *dut*$^-$ *ung*$^-$ strain. Such templates can be used in conjunction with the wide variety of established procedures (gapped duplexes, double priming, etc.) and vectors (single-stranded phage, pBR derivatives, shuttle vectors, etc.). This procedure may also prove to be useful for investigating the mutagenic potential of specific DNA adducts located at defined positions in genes and for studies of mutational specificity of *in vitro* DNA synthesis.[21] The applications of these techniques for engineering DNA sequences and the proteins for which they code are limited only by the need and the imagination of the investigator.

[20] Improved Oligonucleotide-Directed Mutagenesis Using M13 Vectors

By PAUL CARTER

Introduction

Precisely defined mutations may be constructed in DNA fragments cloned into M13 using synthetic oligonucleotides.[1] An oligonucleotide is synthesized which is complementary to part of the DNA template but contains an internal mismatch to direct the required change (point mutation, multiple mutation, insertion, or deletion).[2] The simplest approach to mutagenesis in M13 is "single priming" (Fig. 1), where the mutagenic primer is annealed with the single-stranded M13 template and extended

[1] C. A. Hutchison III, S. Phillips, M. H. Edgell, S. Gillam, P. Jahnke, and M. Smith, *J. Biol. Chem.* **253**, 6551 (1978).
[2] M. Smith, *Annu. Rev. Genet.* **19**, 423 (1985).

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